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The Nuclear Lamina Regulates Germline Stem Cell Niche Organization via Modulation of EGFR Signaling

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SUMMARY

Stem cell niche interactions have been studied extensively with regard to cell polarity and extracellular signaling. Less is known about the way in which signals and polarity cues integrate with intracellular structures to ensure appropriate niche organization and function. Here, we report that nuclear lamins function in the cyst stem cells (CySCs) of *Drosophila* testes to control the interaction of CySCs with the hub. This interaction is important for regulation of CySC differentiation and organization of the niche that supports the germline stem cells (GSCs). Lamin promotes nuclear retention of phosphorylated ERK in the CySC lineage by regulating the distribution of specific nucleoporins within the nuclear pores. Lamin-regulated nuclear epidermal growth factor (EGF) receptor signaling in the CySC lineage is essential for proliferation and differentiation of the GSCs and the transient amplifying germ cells. Thus, we have uncovered a role for the nuclear lamina in the integration of EGF signaling to regulate stem cell niche function.

INTRODUCTION

Many human diseases can be attributed to the disruption of tissue homeostasis. The maintenance and differentiation of stem and progenitor cells, regulated by the niche they reside in, play important roles in supporting tissue homeostasis. Studies in different systems have shown that both extracellular signaling and cell polarity regulate communication between stem/progenitor cells and their niches formed by terminally differentiated tissues or by continuously self-renewing cells. However, it remains unclear how a given signaling pathway or polarity cue is integrated with the nuclear structure to regulate the organization and maintenance of any niche.

The nuclear lamina, which contains the type V intermediate filament proteins (the A- and B-type lamins), forms multiple contacts in the nucleus that link chromatin, nuclear envelope proteins, and nuclear pores to cytoskeleton (Dechat et al., 2008; Simon and Wilson, 2011; Smythe et al., 2000). It therefore represents a potential structural node that could couple cell signaling and polarity to cell morphogenesis. Consistently, lamins have been implicated in the proper development of at least some organs (Coffinier et al., 2010, 2011; Kim et al., 2011; Sullivan et al., 1999; Vergnes et al., 2004; Yang et al., 2011). Studies using tissue culture cells and cell-free assays in the past several decades have shown that lamins could regulate diverse cell functions (Bank and Gruenbaum, 2011; Dechat et al., 2008; Finlan et al., 2008; Goodman et al., 2010; Guelen et al., 2008; Kumaran and Spector, 2008; Ma et al., 2009; Peric-Hupkes et al., 2010; Reddy et al., 2008; Tsai et al., 2006; Zheng, 2010). The relevance for these functions in the context of development and tissue homeostasis, however, remains unknown.

Since the discovery that many mutations in A-type lamins cause human diseases, the effort in studying the disease mechanism has grown exponentially in recent years. Whereas it is critical to use patient samples and animal models to establish how changes in lamins cause human diseases, understanding the basic mechanism that lamins use to regulate organism development and tissue maintenance will also contribute toward the dissection of the disease mechanism. Unfortunately, despite of the existence of lamin mutations and deletions in model organisms such as the mouse, *Drosophila*, and *C. elegans*, the role of lamins in the context of development is still poorly understood.

We reasoned that although many functions have been assigned to lamins, a given cell or tissue type may only use a subset of these functions during certain stages of its differentiation, morphogenesis, and homeostasis. It is therefore important to define such cell- or tissue-specific functions. The *Drosophila* testis is one of the best-characterized systems to study the interaction between stem/progenitor cells and their niches. Well-defined somatic cells form the niche to support germline stem cells (GSCs) and transient amplifying (TA) germline progenitors in the testis. While postmitotic hub cells and cyst stem cells (CySCs) provide the somatic niche for GSCs, the postmitotic cyst cells differentiated from CySCs provide the support for TA germ cells. Cross-signaling among hub cells, GSCs, and CySCs ensures proper maintenance and differentiation of GSCs and CySCs (Davies and Fuller, 2008; de Cuevas and Matunis, 2011; Fuller and Spradling, 2007). For example, epidermal growth factor receptor (EGFR) signaling in the CySC lineage controls the development of the GSC lineage during spermatogenesis (Kiger et al., 2000; Parrott et al., 2012; Sarkar et al., 2007; Tran et al., 2000). Additionally, the hub-cell-secreted ligand induces JAK-STAT signaling in CySCs, which is needed

for proper interactions of the GSCs and CySCs with the hub cells (Cherry and Matunis, 2010; Issigonis et al., 2009; Kiger et al., 2001; Leatherman and Dinardo, 2010; Tulina and Matunis, 2001).

Using the *Drosophila* testis as a model system, we have uncovered a role of lamins in the CySC lineage that form the niche to support the proper proliferation and differentiation of GSCs and TA germline progenitors.

RESULTS

Lamin-B Is Required for *Drosophila* Spermatogenesis

The postmitotic hub cells at the tip of the testis form the niche that directly contacts both the GSCs and CySCs to support their self-renewal and undifferentiated states (Gönczy and DiNardo, 1996; Hardy et al., 1979). Each GSC is encapsulated by a pair of CySCs, and the crosstalk between CySC and GSC is essential for the maintenance and differentiation of both types of stem cells. As the GSC divides to give rise to a progenitor cell called the gonialblast and a new GSC, the pair of CySCs also divides to produce a pair of new CySCs and the differentiating cyst cell pair, which enclose the gonialblast (Figure 1A). As each gonialblast divides and differentiates to form spermatogonia followed by spermatocytes, the cyst cell pair stops dividing and undergoes significant morphological changes, which allow the cyst cells to enclose the TA germline progenitors and spermatocytes (Figure 1A) (de Cuevas and Matunis, 2011; Fuller and Spradling, 2007; Zoller and Schulz, 2012; Lim and Fuller, 2012).

Drosophila expresses one A-type lamin and one B-type lamin called LAMC and Lamin Dm0 (LAM) encoded by *LamC* and *Lam* genes, respectively. We first analyzed the role of LAM during *Drosophila* spermatogenesis. Flies harboring the *Lam* null allele as Lam^{D395}/*Df(2L)cl-h1* transheterozygotes die during larval and pupal stages with ~6% "escapers" that eclose (Muñoz-Alarcón et al., 2007). The surviving adults appeared grossly normal but were male and female sterile. Male sterility could be caused by defects in spermatogenesis. We first focused on the male gonads from the third-instar larvae (L3) using DAPI staining. Since the early germ cells (including GSCs, gonialblasts, and spermatogonia) have more condensed chromatin than the differentiated spermatocytes, their nuclei exhibit brighter DAPI staining than those of the spermatocytes. Thus, DAPI can be used to assess different differentiation stages of the germline (Kiger et al., 2000; Tran et al., 2000). As expected, wild-type L3 male gonads consisted of both early germ cells and differentiated spermatocytes as judged by strong and weak DAPI staining, respectively (Figure 1B) (Kiger et al., 2000; Tran et al., 2000). By contrast, most *Lam* null male gonads were small and were filled with DAPI-bright early germ cells with a near-complete lack of differentiated spermatocytes and spermatids (Figure 1C). Further analyses using antibodies to Notch and Vasa, which label early-stage germ cells and all stage germline cells (Kiger et al., 2000), respectively, confirmed that *Lam* null male gonads were indeed filled with Notch+/Vasa+ early germ cells (Figures 1D–1G).

Next, we used the antibody 1B1 to label the spectrosomes and fusomes, which are spectrin-rich structures found in GSCs, gonialblasts, spermatogonia, and spermatocytes. A single round spectrosome is found next to the centrosome in GSCs and gonialblasts. As a gonialbast undergoes repeated mitosis and incomplete cytokinesis, the spectrosome transforms into an increasingly branched structure called the fusome that connects the sister spermatogonia and spermatocytes through the cleavage furrows in a cyst (Hime et al., 1996). Therefore, the shape of spectrosomes and fusomes can be used as a marker for the early and later germ cells, respectively. We found that wild-type L3 male gonads contained both round spectrosomes and branched fusomes, whereas *Lam* null gonads were filled with early germ cells containing mostly spectrosome-like structures and poorly branched fusome-like structures (Figures 1H and 1I).

To further analyze the germline cells, we used antibodies to phosphorylated histone H3 (pH3) to label the dividing germ cells. Since germ cells in a given cyst divide synchronously, each pH3+ cyst may contain one, two, four, or eight germ cells. We found that in wild-type gonads, 52.2% of the pH3+ cysts contained four or eight germ cells, with the remaining containing one or two germ cells, and all these cysts were localized near the hub (total pH3+ cysts analyzed, 46) (Figure 1J). By contrast, of 538 pH3+ cysts analyzed in the *Lam* null gonads, only 2.4% contained four or more germ cells, with the remaining containing one or two germ cells, and these pH3+ cells were distributed throughout the gonads (Figure 1K). Next, we analyzed male gonads using the *escargot*-lacZ reporter line M5-4 that labels GSCs and gonialblasts. As expected, the *escargot*-lacZ+ GSCs and gonialblasts surrounded the hub in all wild-type gonads analyzed (Figure 1L) and each gonad contained between 5 and 12 escorgot-lacZ+ germ cells (total gonads analyzed, 14). In *Lam* null gonads, however, we found 25 to 157 *escargot*-lacZ+ germ cells per gonads (total gonads analyzed, 12), and many of these cells are far away from the hub (Figure 1M).

We also analyzed the process of GSC differentiation using Bam-GFP (a reporter for spermatogonia). The wild-type spermatogonia cysts formed a tight Bam-GFP-positive band close to the hub as expected (Figure 1N). By contrast, 52.2% (n = 23) of *Lam* null male gonads contained no Bam-GFP-postive spermatogonia (Figure 1O), while the remaining contained large clusters of Bam-expressing germline cells that failed to undergo further differentiation (Figure 1P). No or very few spermatocytes were found in all *lam* null gonads analyzed.

Since ~6% of *Lam* null (Lam^{D395}/*Df(2L)cl-h1*) pupae survive to adulthood, we were able to analyze the adult testis. Compared to wild-type, the 2-day-old *Lam* null testes (11 out of 11) were very small and were filled with DAPI-bright undifferentiated germ cells (Figures 2A and 2E). Using antibodies to Vasa, LAM, and Traffic Jam (TJ, a transcription factor labeling the nuclei of hub cells, CySCs, and early cyst cells) (Li et al., 2003), we detected LAM in both the germline and soma in the wild-type but not in the *Lam* null testis as expected (Figures 2A–2H). The above analyses show that lamin-B is required for the development of *Drosophila* testes.

Lamin-B Functions in the CySC Lineage to Support the Differentiation of GSCs and the Transit-Amplifying Germ Cells

To further understand whether lamin-B functions in the germline or in the soma, we used cell-type-specific RNA interference (RNAi) (Table S1 available online) to knock down LAM. We found that only depleting LAM in the CySC lineage using *tj*-Gal4-mediated RNAi caused defects in GSC differentiation (Figures 2I–2P)

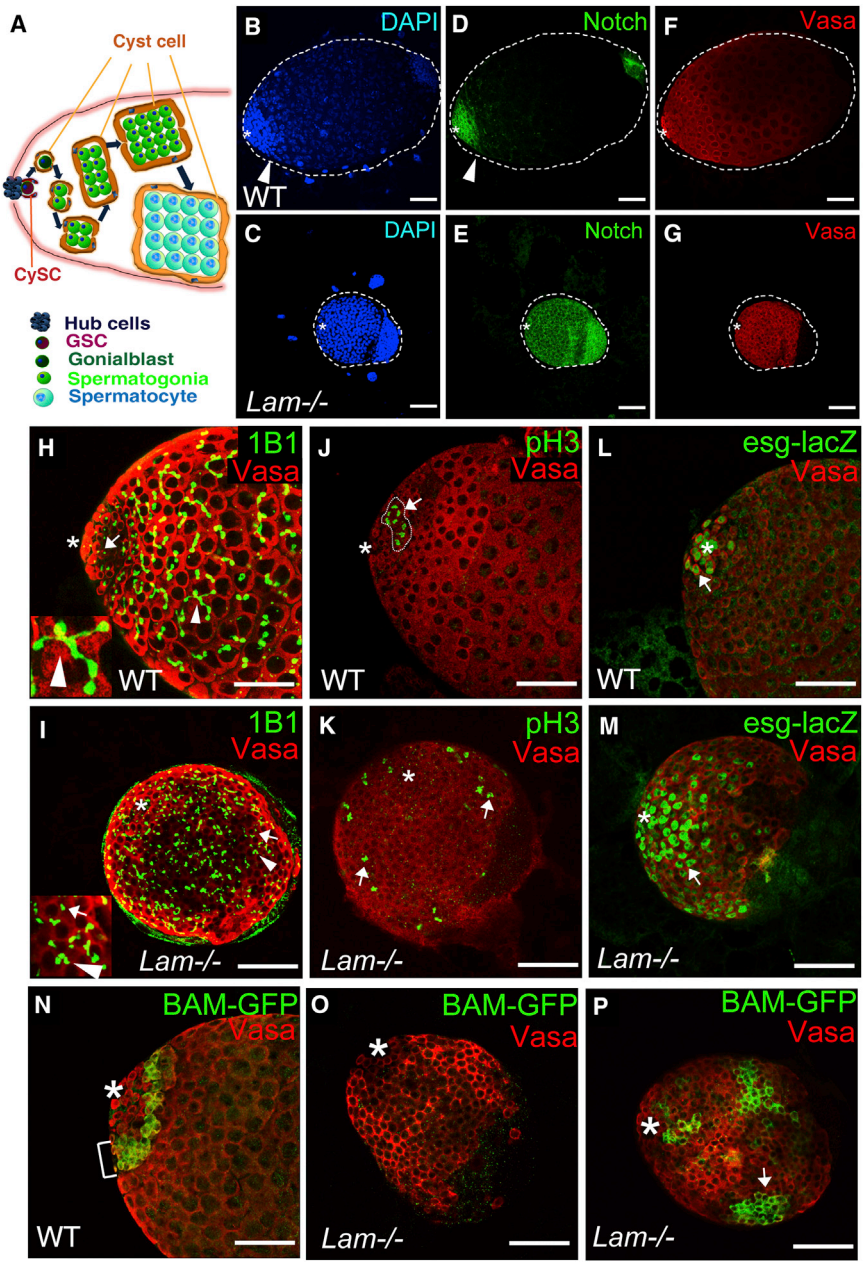

Figure 1. Effects of LAM Deletion on the Differentiation of GSCs and the Transit-Amplifying Germ Cells in Third-Instar Larvae Male Gonads

(A) The hub cells form the niche for both GSCs and CySCs. The asymmetric division of a GSC produces a new GSC and a gonialblast that displace away from the hub along with the pair of cyst cells produced through the asymmetric divisions of a pair of CySCs. Further differentiation of gonialblasts involves spermatogonial mitosis (transient amplification) followed by the growth of spermatocytes, spermatocyte meiosis, and the formation of sperm bundles (not shown). Thick arrows indicate the progression of differentiation and development.

(B–G) Defects of germ cell differentiation in *Lam* null L3 male gonads. DAPI (B and C), Notch (D and E), and Vasa (F and G) staining of wild-type (B, D, and F) and $Lam^{D395}/Df(2L)cl\text{-}h1$ ($Lam^{-/-}$) (C, E, and G) L3 male gonads. Vasa (red) labels germ cells. Notch (green) labels GSCs, gonialblasts, and spermatogonia. Arrowheads mark the transition between spermatogonia and spermatocytes in the wild-type gonad, which is missing in the *Lam* null gonads.

(H and I) L3 Male gonads labeled by the monoclonal antibody 1B1, which stains the spectrin in spectrosomes and fusomes (green). The wild-type gonad (H) contained both spherical spectrosomes (in GSCs, gonialblasts, and two-cell stage spermatogonia, arrow) and branching fusomes (in spermatogonia and spermatocytes, arrowhead), while the *Lam* null gonad (I) contained mostly spectrosome-like structures (arrow) and a few poorly branched fusomes (arrowhead). The inset in (H) shows an enlarged area with the arrow pointing to a branched fusome. The inset in (I) shows an enlarged area with the arrow and arrowhead pointing to a spectrosome and a poorly branched fusome, respectively.

(J and K) A wild-type L3 male gonad (J) contains a cyst of eight pH3+ germ cells (arrow) that is close to the hub, whereas most pH3+ germ cells in the *Lam* null gonad (K) appear as singlets or pairs (arrows) and many of them are far away from the hub (asterisk).

(L and M) Labeling of hubs (asterisks), GSCs, and gonialblasts by $escargot^{M5\text{-}4}$-LacZ (green) revealed the accumulation of undifferentiated GSCs or gonialblasts (arrows) in the *Lam* null male gonad (M) compared to the wild-type control (L).

(N–P) Labeling of spermatogonia by BAM-GFP expression indicates the expected localization of BAM-GFP+ cells in wild-type (N) close to the hub (asterisks). In the *Lam* null gonads, 52.2% (n = 23) do not have BAM-GFP+ cells (O), while the remaining gonads (P) contain large clusters of BAM-GFP+ cells that are displaced away from the hub. The arrow in (P) marks a cluster of more than 16 BAM-GFP+ germ cells.

Scale bars represent 50 μm.

similar to those seen in *Lam* null testes (compare to Figures 2A–2H). Importantly, using cell-type-specific Gal4 to express *Lam* complementary DNA (cDNA) in the germline lineage, hub cells, or CySC lineage, we found that only the CySC-lineage-specific expression of LAM fully rescued GSC differentiation defects (Figures 2Q–2X) and male fertility (26 out of 26 testes analyzed) in *Lam* null survivors (Table S1). Additionally, the germline mitotic clones of Lam^{D395} homozygotes produced cysts containing differentiating spermatocytes (Figure S1) and spermatids (not shown). Thus, LAM is required in the CySC lineage but is dispensable in both the germline and the hub cells.

Lamin-B Functions in CySCs to Ensure Proper Interactions of CySCs and GSCs with the Hub

Although both CySCs and GSCs interact with the postmitotic hub cells, the pair of CySCs surrounding each GSC only contact the hub through the long and thin cellular protrusions with their

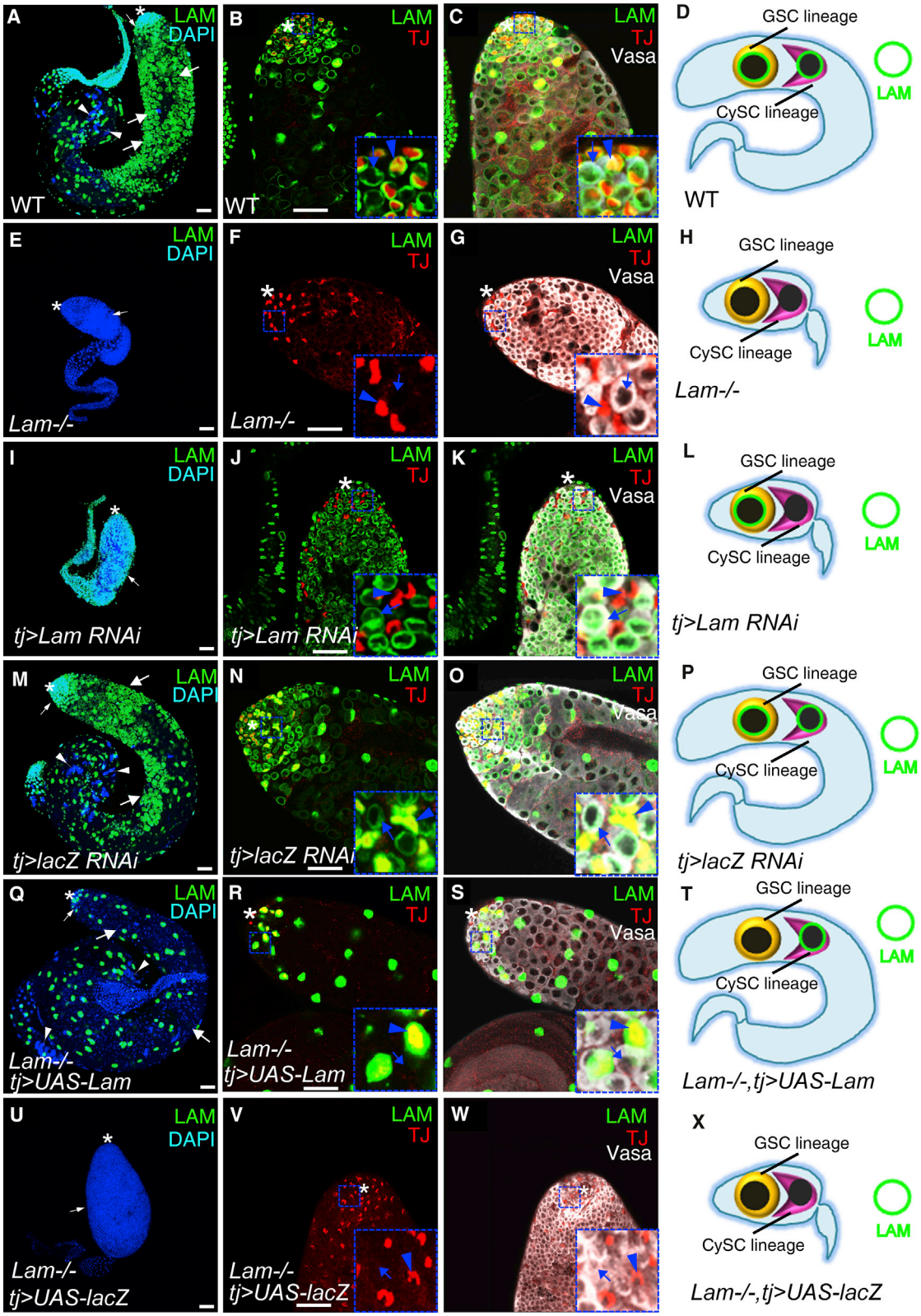

Figure 2. LAM Functions in the CySC Lineage to Support the Differentiation of GSCs and the Transit-Amplifying Germ Cells

(A–H) Whole-mount testes from 2-day-old wild-type and *Lam* null animals stained with DAPI and antibodies to LAM, TJ (the CySC lineage), and Vasa (the germline). The tip of wild-type (A) and *Lam* null (E) testes are enlarged in (B) and (C) and (F) and (G), respectively. (C) and (G) show the merge of triple-color label of LAM, TJ, and Vasa of the same testes shown in (B) and (F), respectively. Small white arrows in (A) and (E) show early germ cells. Large white arrows and

(legend continued on next page)

nuclei displaced away from the hub and appearing juxtaposed at the base of the GSCs (Figures 3A and 3B) (Issigonis et al., 2009; Tarayrah et al., 2013). Using Zfh-1, a transcription factor strongly expressed in the nuclei of CySCs and weakly expressed in the hub and early cyst cells (Leatherman and Dinardo, 2008), we found that compared to wild-type L3 male gonads, the nuclei of many CySCs in *Lam* null gonads were positioned adjacent to the hub cells (Figures 3C and 3E). A similar phenotype was observed in *tj*-driven *Lam* RNAi L3 male gonads (Figures 3D and 3E) in which LAM was depleted only in the CySC lineage. We found that the total number of Zfh-1 positive CySC lineage cells was similar among the wild-type control (32.5 ± 5.5, n = 13), the *tj*-driven *LacZ* RNAi control (31.9 ± 2.1, n = 14), *Lam* null (34.6 ± 6.2, n = 17), and *tj*-driven *Lam* RNAi (35.1 ± 8.1, n = 18; Student's t test, $p > 0.05$) gonads. Thus, LAM regulates the localization of CySCs within the niche but not their proliferation.

Previous studies have shown that increasing the contact between the CySCs and the hub can lead to the displacement of GSCs from the hub (Issigonis et al., 2009; Leatherman and Dinardo, 2010). We found that both *Lam* null and *tj*-driven *Lam* RNAi male gonads have a reduction of the number of GSCs contacting the hub compared to controls (Figures 3B–3D and 3F). Therefore, lamin-B is needed in CySCs to regulate the organization of the niche so that both the CySCs and GSCs can assume proper interactions with the hub.

Lamin-B Is Required for the Morphogenesis of the Differentiating Cyst Cells

The above findings suggest that the CySCs may not undergo proper differentiation to form cyst cells. We analyzed the early differentiating cyst cells in detail and found that whereas the TJ+ cyst cells enclosed the germline in developing wild-type L3 male gonads as expected, cyst cells in *Lam* null gonads were displaced away from germ cells, with many cyst cells mislocalized to the surface of the developing gonads (Figures 3G, 3H, and S2). Using the Flp-out RNAi strategy to specifically mark individual LAM-depleted cyst cells by GFP, we found that while the control cyst cells extended their cell bodies to surround the germline cyst, the cell bodies of LAM-depleted cyst cells remained compacted (Figures 3I and 3J). Thus, LAM is required in both the CySCs and their differentiating cyst cells to ensure proper interactions of the GSCs and the TA germline cells with their respective niches (Figure 3K).

Lamin-B Regulates Nuclear EGFR Signaling in CySCs and Cyst Cells

The testis phenotypes observed in *Lam* null flies are reminiscent of those seen in cyst cells defective in the EGFR signaling pathway (Kiger et al., 2000; Sarkar et al., 2007; Schulz et al., 2002; Tran et al., 2000). EGFR signals both in the nucleus and cytoplasm. Whether it is the nuclear or cytoplasmic EGFR signaling that is required in cyst cells is unknown (Gabay et al., 1997; Kumar et al., 1998; Lesokhin et al., 1999). Our observation suggests that lamin-B could promote the nuclear EGFR signaling in both CySCs and the differentiating cyst cells. We found that overexpression of *Lam* cDNA in EGFR pathway mutants failed to rescue testis defects (data not shown). Since the nuclear translocation of phosphorylated ERK (dpERK) is important for nuclear EGFR signaling, we analyzed dpERK localization in CySCs and cyst cells. We found that in wild-type L3 male gonads, dpERK was localized in both the nucleus and cytoplasm of almost all of the CySCs and early cyst cells (Figures 4A and 4C). By contrast, dpERK remained largely in the cytoplasm in most of the CySCs (Figure 4B) and early cyst cells in *Lam* null gonads (Figure 4D).

Since EGFR signaling is also required in the follicle cells for oogenesis in female flies (Nilson and Schüpbach, 1999; Zhao et al., 2000), we analyzed whether lamin-B is required for dpERK nuclear translocation in these cells. We used FLP/FRT-mediated mitotic recombination to generate Lam^{D395} mutant follicle cell clones. The recombination leading to Lam deletion also removes GFP that marks the wild-type follicle cell nuclei. Thus, the follicle cells not marked by GFP are null for lamin-B. We found that dpERK was only localized in the GFP-positive nuclei of the *Lam* wild-type follicle cells (Figure S3). Therefore, LAM is also required for the nuclear localization of dpERK in these cells. Consistent with this, depletion of lamin-B in follicle cells resulted in female sterility (data not shown). Taken together, lamin-B is required for the nuclear EGFR signaling in CySC, cyst cells, and follicle cells that rely on this pathway.

Lamin-B-Mediated Nuclear ERK Localization in CySC Is Required for Proper Cell-Cell Interactions

To confirm that lamin-B indeed regulates the CySC lineage through ERK, we depleted either *Drosophila* ERK (dERK) or *Drosophila* MAPKK (DSOR1) in the CySC lineage using *tj*-driven RNAi. Similar to LAM, dERK and DSOR1 function in the CySC lineage to promote the differentiation of the germline (Figures

arrowheads in (A) show spermatocytes and sperm bundles, respectively. Insets in (B) and (C) and in (F) and (G) show enlarged areas with blue arrows and arrowheads indicating Vasa+ germ cells and TJ+ cyst cells, respectively. (D) and (H) illustrate the nuclear LAM (green circle) status in the GSC and CySC lineages in wild-type (D) and *Lam* null (H) testes. For clarity, only one GSC and one CySC are shown. *Lam* null in both lineages leads to the formation of small testes as shown in (H).

(I–P) Testes with the CySC lineage depletion of LAM (I–L) using the *tj*-Gal4-driven *Lam* RNAi allele (v45635), but not the control LacZ RNAi (M–P), phenocopied defects seen in *Lam* null testes (see E–H). The tip of the testis treated by *Lam* RNAi (I) or control RNAi (M) is enlarged in (J) and (K) and in (N) and (O), respectively. (K) and (O) show the merge of triple color label of LAM, TJ, and Vasa of the same testes shown in (J) and (N), respectively. Blue arrows and arrowheads in the enlarged insets in (J) and (K) and in (N) and (O) indicate Vasa+ germ cells and TJ+ cyst cells, respectively. (L) and (P) illustrate the testes with cyst cell depletion of LAM by RNAi (L), but not control RNAi (P), phenocopied the defects seen in *Lam* null testes.

(Q–X) Expression of *Lam* cDNA in the CySC lineage using *tj*-Gal4 (Q–T), but not control *LacZ* cDNA (U–-X), fully rescued the testis defects and male fertility in the *Lam* null mutants. The tip of a wild-type (Q) and *Lam* null (U) testes are enlarged in (R) and (S) and in (V) and (W), respectively. (S) and (W) show the merge of triple-color label of LAM, TJ, and Vasa of the same testes in (R) and (V), respectively. The enlarged insets in (R) and (S) and in (V) and (W) show Vasa+ germ cells (lack LAM, blue arrows) and TJ+ cyst cell (LAM+ in R and S or LAM− in V and W, blue arrowheads). (T) and (X) illustrate that *Lam* cDNA expression, but not the control LacZ cDNA, in the CySC lineage is sufficient for rescuing the testis defects in *Lam* null mutants.

Asterisks represent hubs. Scale bars represent 50 μm. See also Figure S1 and Table S1.

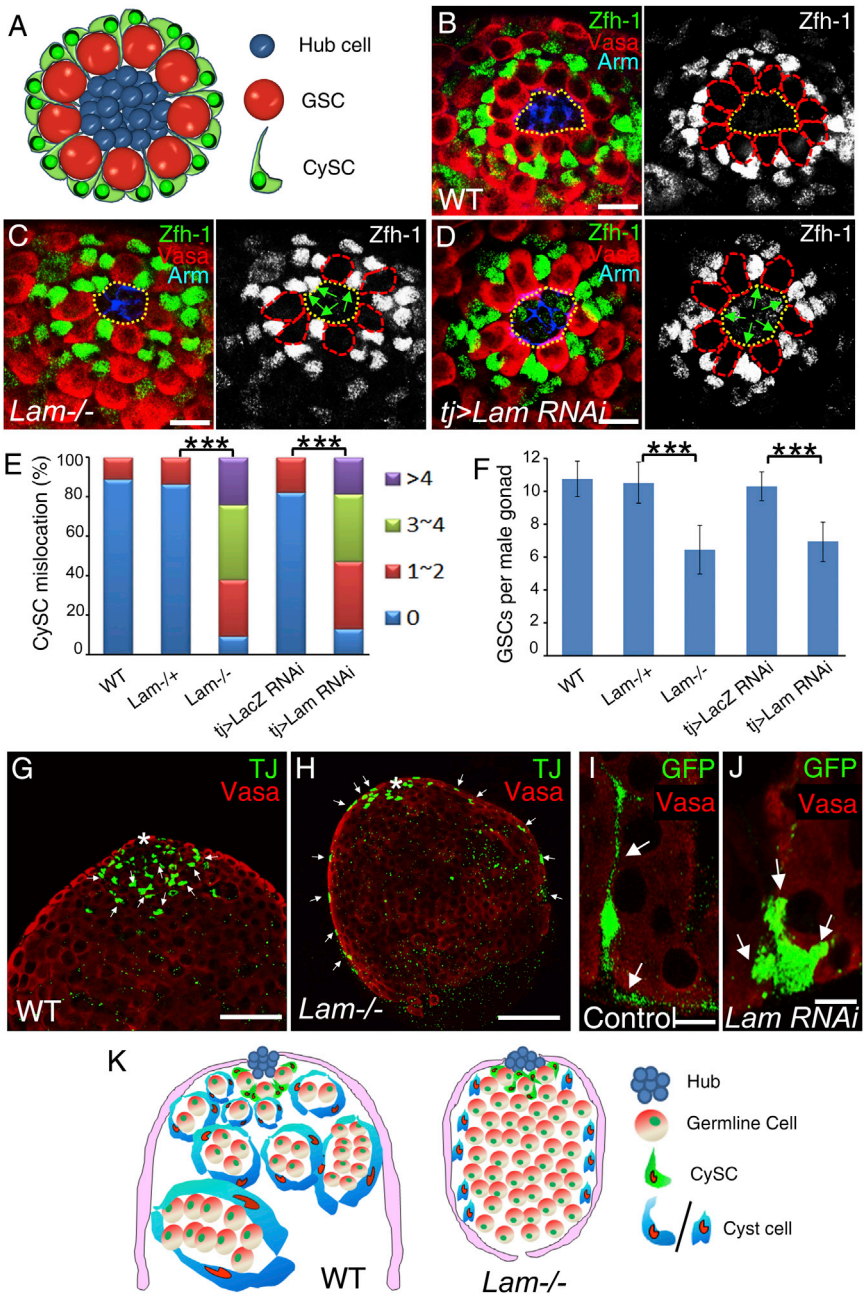

Figure 3. LAM Regulates CySC Localization and Cyst Cell Morphogenesis

(A) A cartoon of the testis niche illustrating the organization of GSCs and CySCs with respect to the hub cells. In wild-type testes, Zfh-1-expressing CySCs surround GSCs. The thin cellular protrusions of CySCs contact the hub with the nuclei localized at the base of the GSCs away from the hub.

(B–D) Immunostaining using antibodies against *Drosophila* beta-catenin (Arm, blue), Vasa (red), and Zfh-1 (green or white) in wild-type (B), *Lam* null (C), and Tj > *Lam* RNAi L3 male gonads (D). Green arrows point to the Zfh-1-expressing CySCs with their nuclei localized close to the hub in (C) and (D), while such kind of close localization is not seen in the control testis (B). The hubs are outlined by yellow dotted lines, while GSCs are outlined by red dotted lines in the images with only Zfh-1 staining. Scale bars represent 10 μm.

(E) Quantification of the percentage of L3 male gonads with mislocalized CySCs as judged by the close proximity of the Zfh-1+ nuclei to the hub as seen in (C) and (D). The colored bars indicate the number of CySCs showing mislocalization in each hub. LAM null (n = 36) and tj > LAM RNAi (n = 36) L3 male gonads exhibit a significant increase of mislocalized CySCs compared to the wild-type (n = 27), *Lam* heterozygous (n = 31), or LacZ RNAi (n = 28) controls. ***p < 0.001, Wilcoxon two-sample test.

(F) LAM null (n = 21) and tj > LAM RNAi (n = 23) L3 male gonads exhibit a significant decrease in the number of GSCs associated with the hub compared to the wild-type (n = 16), *Lam* heterozygous (n = 18), or LacZ RNAi (n = 17) controls. ***p < 0.001, Student's t tests. Error bars represent SD.

(G and H) The cross-section views of L3 male gonads. Compared to wild-type, the TJ+ (green, indicated by white arrows) cyst cells in the *Lam* null gonad were displaced away from the early germ cells (red, Vasa) near the hub with many cyst cells mislocalized to the surface of the gonads. Asterisks represent hubs. Scale bar represents 50 μm.

(I and J) The use of the Flp-out RNAi strategy to specifically mark individual cyst cells by GFP. UAS-GFP expressed in both the cytoplasm and nucleus labeled the control (I) and *Lam*-RNAi (J) cyst cells. Antibodies to GFP (green) and Vasa (red) were used. The long cell extensions seen in the control cyst cell (arrows in I) are missing in the *Lam*-RNAi cyst cell (arrows in J). Scale bars represent 20 μm.

(K) Cartoon illustration of the role of lamin-B in regulating the CySCs localization and the morphogenesis of cyst cells.

See also Figure S2.

5A–5D). Additionally, a stronger defect in germline differentiation was observed upon dERK depletion in cyst cells lacking one wild-type copy of *Lam* than in those with two copies (compare Figure 5E to Figure 5C). These data suggest that LAM promotes EGFR signaling in the CySC lineage by regulating nuclear translocation/retention of dpERK and subsequent pathway activation in the nucleus during spermatogenesis.

Next, we analyzed whether depleting dERK and DSOR1 using *tj*-driven RNAi would disrupt the interactions of CySCs and GSCs with the hub as seen in the *Lam* null gonads. Compared to the control RNAi (*tj*-driven *LacZ* RNAi), depletion of either dERK or DSOR1 in the CySC lineage resulted in increased interactions between the CySC and the hub and a corresponding decrease in the number of GSCs contacting the hub (Figures 5F, 5G, 5N, 5O, and S4A), similar to those seen in the *Lam* null or *Lam*-RNAi gonads (Figures 3B–3F). Again the overall numbers of the CySC lineage cells (judged by Zfh-1 staining) per gonad were similar (p > 0.05) among control *tj*-driven *LacZ* RNAi

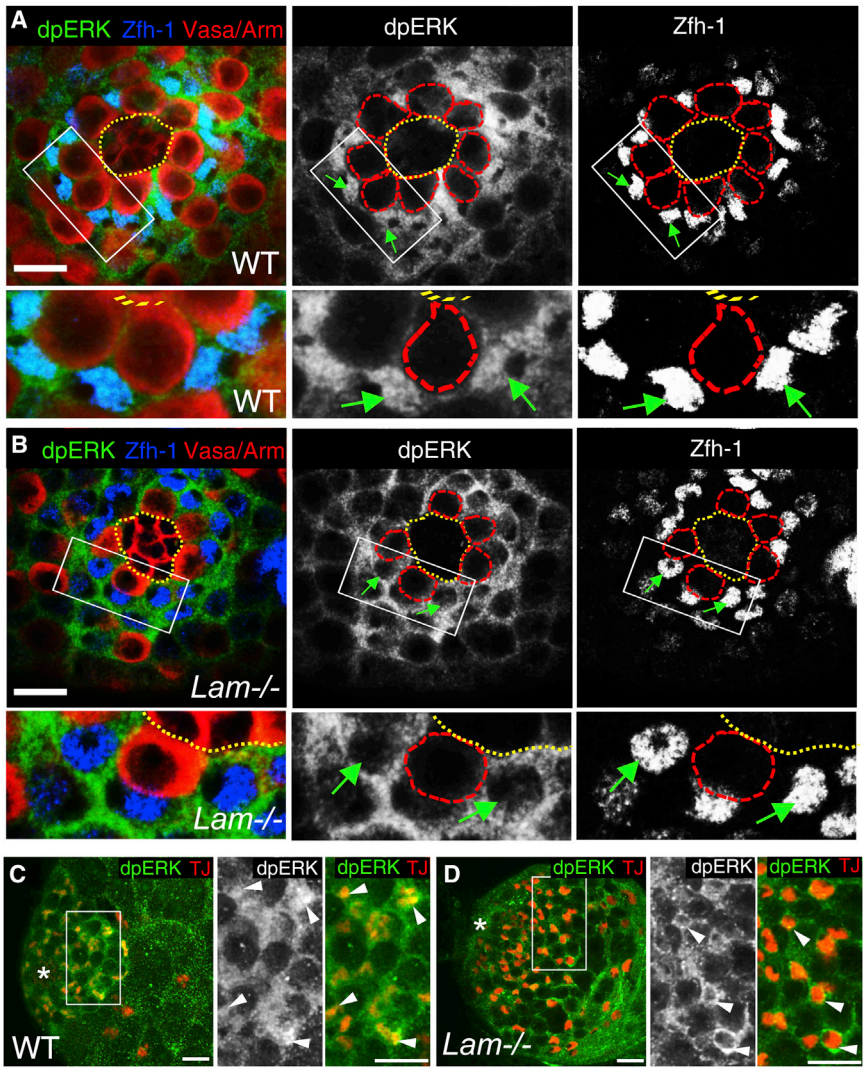

Figure 4. LAM Regulates dpERK Nuclear Location in CySCs and Cyst Cells

(A and B) dpERK is localized in both the cytoplasm and the nuclei of CySCs (blue, Zfh-1) in wild-type (A) but is depleted in the nuclei of CySCs in *Lam* null (B) L3 male gonads. The boxed areas in (A) and (B) are enlarged at the bottom. Green arrows indicate the CySCs with (A) or without (B) nuclear dpERK staining. Immunostaining using antibodies against dpERK (green), Arm (red, labels the hub), Vasa (red), and Zfh-1 (blue) in wild-type (A) and LAM null (B) L3 male gonads is shown. Hub areas are outlined with yellow dotted lines, while GSCs in the black and white images are outlined with red dotted lines. Scale bars represent 10 μm.

(C and D) dpERK is enriched in the cyst cell nuclei (red, TJ) in wild-type (C), but not in *Lam* null (D), L3 male gonads. The boxed areas in (C) and (D) are enlarged to the right. Arrowheads show cyst cells with (C) or without (D) nuclear dpERK staining. Asterisks represent hubs. Scale bars represent 20 μm.

See also Figure S3.

(33.1 ± 5.3, n = 16), *dERK* RNAi (33.8 ± 7.2, n = 17), and *Dsor1* RNAi (34.6 ± 6.5, n = 17).

To directly test the role of LAM in the nuclear translocation of dpERK in the CySC lineage, we linked a nuclear localization signal (NLS) in frame to the C terminus of dERK and used the *tj* promoter to express it in the CySC lineage depleted of LAM by RNAi. We found that this partially rescued the GSC differentiation defect as revealed by the formation of spermatocytes and sperm bundles (Figures 5H–5K) in 45.2% of testes (n = 42). Although the remaining testes still had a large number of early germ cells (Figures S4B and S4C), they were fewer compared to LAM-depleted testes rescued with LacZ controls (compare Figures S4B and S4C to Figures 5J and 5K). Despite the formation of sperm bundles, forced expression of dERK-NLS failed to rescue fertility (data not shown). Additionally, we observed a strong rescue in 40.6% of the *Lam* null L3 male gonads (n = 32) by expressing dERK-NLS in the CySC lineage. These rescued gonads formed spermatocytes and did not accumulate many early germline cells (compare Figures S4E and S4E′ to Figures S4F and S4F′). Although the remaining L3 male gonads still accumulated early germ cells (Figures S4G and S4G′), the degree of accumulation was less compared to *Lam* null gonads (compare Figures S4G and S4G′ to Figures S4E and S4E′). The size of all of the rescued gonads appeared smaller than wild-type gonads, but they all contained spermatocytes. Thus, the block of spermatogonia to spermatocyte transition due to lamin-B depletion can be rescued by forced expression of nuclear ERK in the CySC lineage. Importantly, forced expression of dERK in the CySC nuclei resulted in a significant reversal of the interaction defects between CySCs and GSCs with the hub, which corresponded to the increased number of GSCs contacting the hub as compared to the *Lam* null gonads (Figures 5L–5O). Our findings demonstrate that LAM promotes GSC-hub interaction and GSC differentiation at least in part by facilitating dERK nuclear localization in the CySC lineage.

Previous studies show that the adhesion between the CySC and the hub is mediated by both E-cadherin and βPS-integrin (Issigonis et al., 2009; Voog et al., 2008). We analyzed whether lamin-B is required in the CySCs to prevent excess expression of E-cadherin and βPS-integrin. E-cadherin expression was similar in wild-type and *Lam* null L3 male gonads (Figures S4H and S4I). The expression of βPS-integrin in CySCs was, however, increased in *Lam* null gonads compared to controls (Figures S4J and S4K). Thus lamin-B-regulated EGFR signaling could limit βPS-integrin-mediated CySC and hub interactions.

Lamin-B Regulates Nuclear EGFR Signaling through Nucleoporins

In mammalian tissue culture cells, the nuclear translocation and retention of phosphorylated ERK require nucleoporins (NUP) 153

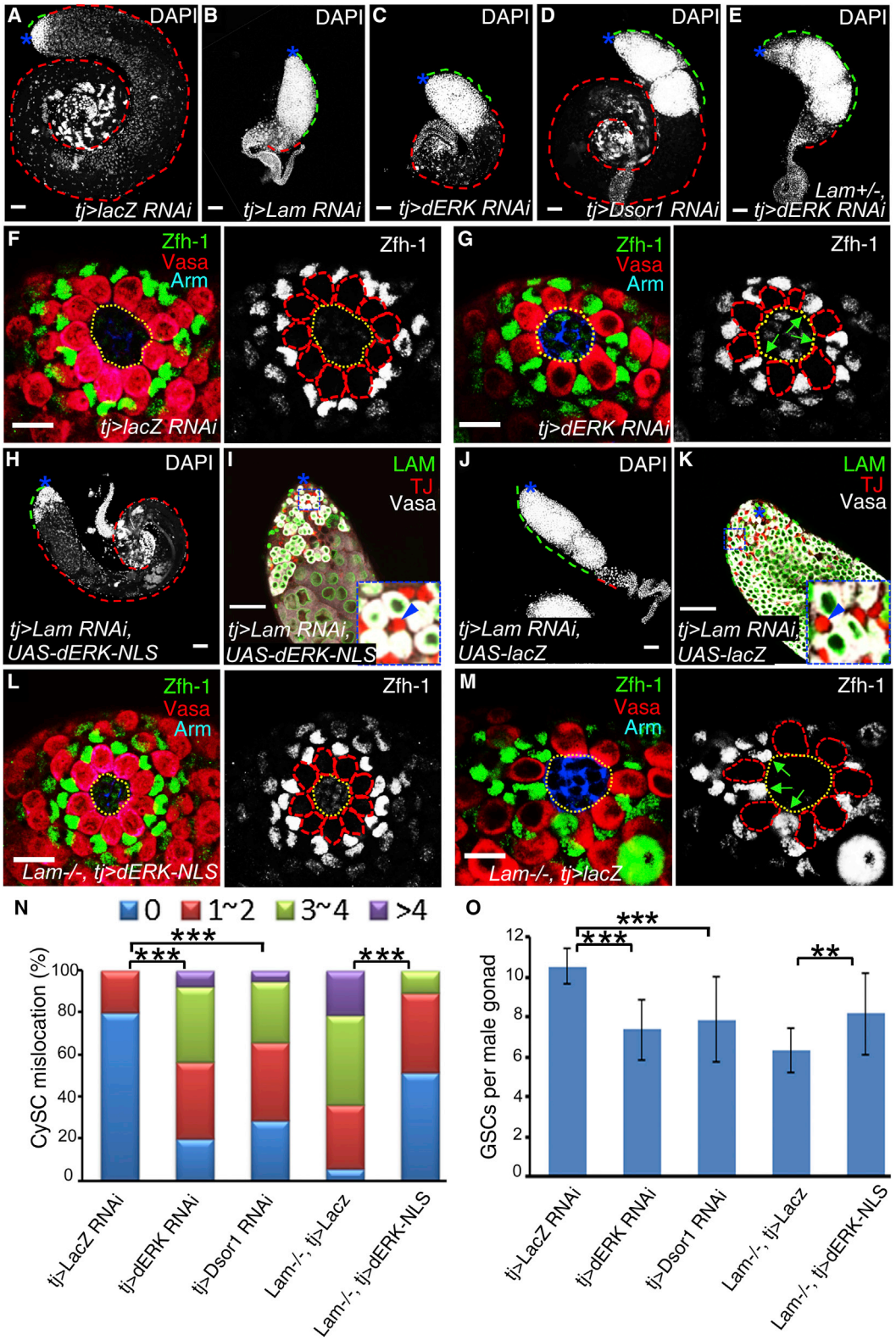

Figure 5. LAM Regulates GSC Maintenance and Differentiation in the CySC Lineage through EGFR Signaling

(A–E) The CySC lineage depletion of dERK (C) or DSOR1 (D) by *tj*-driven RNAi phenocopied the CySC lineage depletion of LAM (B) as revealed by the accumulation of DAPI-bright early germ cells compared to control LacZ RNAi (A). Cyst cell depletion of dERK in $Lam^{+/-}$ (E) resulted in a more severe phenotype than in $Lam^{+/+}$ flies (compare E to C). Green and red dashed lines outline the DAPI-bright early and DAPI-dim late germ cells, respectively. Scale bars represent 50 μm.

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and TPR, which are known to bind to one another (Adachi et al., 1999; Lorenzen et al., 2001; Matsubayashi et al., 2001; Smith et al., 2010; Vomastek et al., 2008; Whitehurst et al., 2002). Additionally, in vitro studies suggest that lamins bind and regulate NUP153 localization in the nuclear pore complex (NPC). However, the physiological significance of these observations is unknown in any in vivo setting. We reasoned that LAM might regulate dpERK through NUP153 and TPR in both CySCs and cyst cells. Consistent with this, we found that *Drosophila* NUP153 interacts with LAM (Figure 5SA). In wild-type testes, NUP153, MTOR (the *Drosophila* TPR), and NUP98 exhibited even distributions around the nuclear rim in the GSCs, TA germline cells, CySCs, and cyst cells (Figures 6A–6C and S5B–S5E). LAM depletion in the CySC lineage caused aggregation of NUP153 and MTOR without affecting the distribution of NUP98 in these cells (Figures 6D–6F and S5F–S5H). Additionally, we analyzed the *Lam* null mitotic follicle cell clones in female ovaries and found that LAM was also required for proper localization of NUP153 and MTOR but not NUP98 and NUP154 (Figures S5I–S5L).

Consistent with the normal import of nuclear proteins such as TJ and NLS-GFP in the LAM-depleted CySC lineage (not shown), electron microscopy analyses revealed that NPCs in these cells were indistinguishable between *Lam*-depleted and control testes (Figures 6G and 6H). Therefore, instead of regulating the general NPC structure and function, LAM is specifically required for dpERK nuclear translocation and/or retention by ensuring proper NPC localization of NUP153 and MTOR in the CySC lineage. Supporting a role of NUP153 and MTOR in EGFR signaling in these cells, depletion of either protein in these cells caused a similar defect in germline differentiation as seen in *Lam* mutants (Figures 6I and 6J; compare to Figure 5B). Importantly, we found that the CySCs depleted of either NUP153 or MTOR exhibited an increased interaction with the hub, which corresponded to a decrease of the number of the hub-associated GSCs (Figures 6K and 6L), but the number of CySCs was not affected (data not show). Thus, lamin-B regulates EGFR signaling in the CySC lineage by controlling the proper distribution of NUP153 and MTOR in the NPCs.

A- and B-type Lamins Have Shared Functions

Since a given animal cell often expresses both A- and B-type lamins, a critical question in the field is whether different lamins can compensate one another for certain functions in vivo. This is particularly relevant with regard to the role of lamins in regulating NUP153, because both A- and B-type lamins can bind to this nucleoporin (Al-Haboubi et al., 2011; Smythe et al., 2000). Immunofluorescence analyses show that while LAM is express in all cell types in the testes (Figures 7A and S6A), LAMC is only expressed in the hub cells and late-stage germline and cyst cells but is undetectable in the early stage germ cells (including GSCs, gonialblasts, and spermatogonia), the surrounding CySCs, and early cyst cells (Figures 7B and S6B). Similarly, whereas LAM is expressed in the follicle cells in the early-stage ovaries, LAMC is undetectable in these cells (Figures S6C and S6D). The differential expression of lamins coupled with the strong defects observed in cells only expressing LAM offer us a unique opportunity to test whether LAM and LAMC have shared functions.

We found that ectopic expression of LAMC cDNA using *tj*-Gal4 in the CySC lineage partially rescued the germ cell differentiation defects in 58.9% of LAM-depleted testes (Figures 7C and 7D; compare to Figures S6G and S6H). These rescued testes were shorter (~1.4 mm) compared to controls (~2.0 mm), but they contained germ cells at all stages of differentiation. Although the remaining 41.1% testes appeared much shorter (~0.8 mm) and abnormal, they had less severe accumulation of early germ cells compared to controls, suggesting a weak rescue (Figures S6E and S6F). As expected, LAMC expression also partially rescued the NUP153 location defects (Figure 7E; compare to Figure S6I), which corresponded to a partial rescue of nuclear dpERK localization in the CySCs depleted of LAM (Figure 7F; compare to Figure S6J). Therefore, A- and B-type lamins have a shared function in nuclear EGFR signaling by ensuring proper NUP153 localization.

DISCUSSION

The difficulty in understanding the role of lamins in the context of development and disease has been confounded by the diverse

(F and G) The CySC lineage depletion of dERK by *tj*-driven *dERK* RNAi (G), but not the control *lacZ* RNAi (F), phenocopied the CySCs mislocalization observed in *Lam* null gonads. Arm (blue), Vasa (red), and Zfh-1 (green) label the hub, the germline lineage, and the CySC lineage, respectively. Green arrows in the black and white image in G point to the Zfh-1-expressing CySCs with their nuclei close to the hub. The hubs are outlined with yellow dotted lines, while GSCs (in the black and white images) are outlined with red dotted lines. Scale bars represent 10 μm.

(H–K) The effects of forced expression of dERK-NLS in the CySC lineage depleted of LAM on spermatogenesis. An example of a strongly rescued testis is shown in (H) and (I) (19 out of 42) along with the control LacZ rescued testis (J and K). The testis tips in (H) and (J) are enlarged in (I) and (K), respectively, with the enlarged insets showing the expression of LAM (green) in germ cells (Vasa+, white) but not in cyst cells (blue arrowheads pointing to the TJ+, red cyst cells). Asterisks represent hubs. Scale bars represent 50 μm.

(L and M) Forced expression of dERK-NLS (L), but not LacZ (M), in the CySC lineage, effectively rescued the CySC mislocalization defect in *Lam* null L3 male gonads. Hubs, germline cells, CySCs are labeled by Arm (blue), Vasa (red), and Zfh-1 (green), respectively. Green arrows in the black and white image in (M) point to the Zfh-1+ CySCs with their nuclei next to the hub. The hubs are outlined with yellow dotted lines, while GSCs (in the black and white images) are outlined with red dotted lines. Scale bar represents 10 μm.

(N) Quantification of the percentages of L3 male gonads with mislocalized CySCs as judged by the close proximity of the Zfh-1+ nuclei to the hub. The colored bars indicate the number of CySCs showing mislocalization in each hub. The *tj*-driven *dERK* (n = 35) or *Dsor1* RNAi (n = 32) caused a significant increase of gonads with mislocalized CySCs compared to the control *tj*-driven LacZ RNAi (n = 30). The *tj*-driven expression of dERK-NLS in *Lam* null gonads (n = 25) resulted in a significant rescue of CySC localization defects compared to control *tj*-driven LacZ expression (n = 26). ***$p < 0.001$, Wilcoxon two-sample test.

(O) Quantification of the number of GSCs associated with the hub. The *tj*-driven *dERK* (n = 21) or *Dsor1* RNAi (n = 19) caused a significant decrease in the number of GSCs associated with the hub compared to the control *tj*-driven *LacZ* RNAi (n = 16). The *tj*-driven expression of dERK-NLS in *Lam* null animals (n = 15) resulted in a significant increase in the number of GSCs associated with the hub compared to the control *tj*-driven LacZ expression (n = 14). **$p < 0.01$, ***$p < 0.001$, Student's t tests. Error bars represent SD.

See also Figure S4.

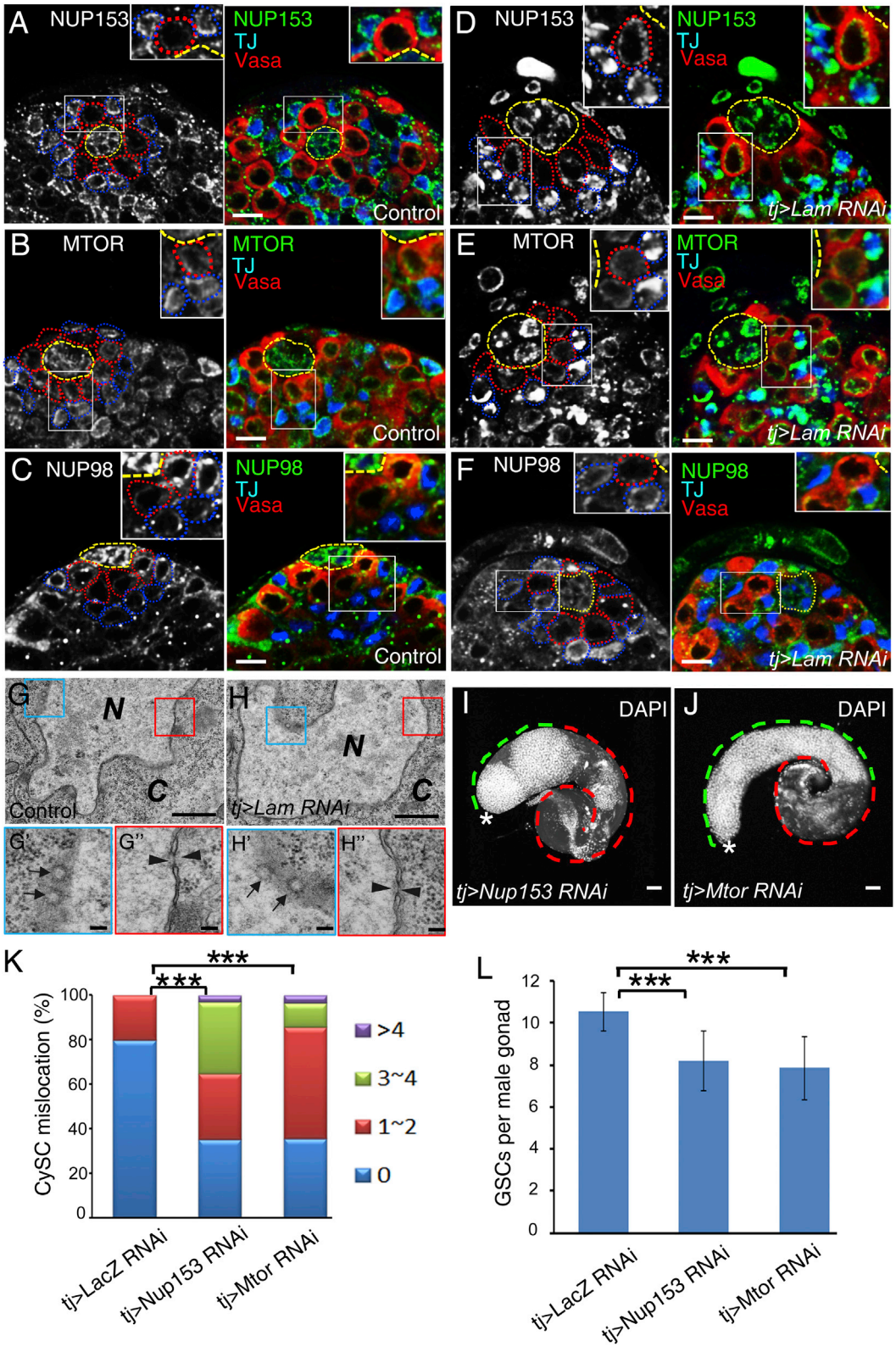

Figure 6. LAM Regulates EGFR Signaling through NUP153 and MTOR

(A–F) NUP153 (A), MTOR (B), and NUP98 (C) are evenly distributed throughout the nuclear envelope of both CySCs (TJ, red) and GSCs in adult testes treated with control RNAi (*tj*-Gal4 > *lacZ* RNAi), whereas LAM depletion in CySCs (*tj*-Gal4 > *Lam* RNAi) caused aggregation of NUP153 (D) and MTOR (E) without affecting NUP98 (F) in CySCs. Insets show enlarged images revealing in more detail the distribution of nucleoporins in CySCs. The bright dots in (C)

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functions ascribed to these proteins based on in vitro studies. Recent efforts using animal models and embryonic stem cells have shown that B-type lamins are required for proper building of at least certain organs (Kim et al., 2012), although how lamins regulate organogenesis is unknown. The lamin-regulated EGFR signaling in CySCs and cyst cells we uncover here (Figure 7G) represent a molecular mechanism for these proteins in promoting development.

It has been known for some time that lamins are required for the even distribution of nuclear pores in the nuclear envelope in tissue culture cells in vitro. However, the physiological significance of such a function has remained unknown. Also unclear is the in vivo relevance of Nup153-regulated nuclear translocation/retention of phosphorylated ERK. Our findings demonstrate that lamin-regulated Nup153 distribution is critical for proper nuclear retention of dpERK in CySC lineage and follicle cells, which are two somatic cell types that we find to exhibit strong nuclear dpERK staining in male and female reproductive systems, respectively. Interestingly, similar to the effect of lamin-B depletion in the CySC lineage in males, the follicle cells depleted of lamin-B also leads to oogenesis defects and female sterility (data not shown). Therefore, our findings strongly suggest that lamin is required in the niche or microenvironment to support germline development in both sexes. It will be important to further study whether lamins have a general role in regulating cells in the niche that requires nuclear EGFR signaling and whether such function could contribute toward diseases caused by lamin mutations in humans.

We have shown that lamin- and nucleoporin-regulated EGFR signaling in the nuclei of CySCs is important to limit the interaction between CySCs and hub cells. Depletion of lamin-B, NUP153, MTOR, ERK, or MAPKK leads to increased CySC-hub interactions and a corresponding decrease of hub-associated GSCs. Importantly, this defect can be rescued by forced nuclear expression of ERK in CySCs depleted of lamin-B. CySCs depleted of lamin-B exhibited stronger expression of βPS-integrin than that of the wild-type CySCs. Previous studies have shown that the expression of SOCS36E, the JAK/STAT-induced inhibitor that suppresses the JAK/STAT pathway, inhibits the expression of βPS-integrin in CySCs (Issigonis et al., 2009). Since EGFR signaling can activate SOCS36E in certain cell types in *Drosophila* (Herranz et al., 2012), we speculate that the lamin-B-regulated EGFR signaling might control the βPS-integrin-mediated interaction between CySCs and the hub via the SOCS36E regulatory network. Further studies aimed at understanding how lamin-B and nucleoporin-regulated EGFR signaling controls integrin expression in CySC cells should shed light on the role of the nuclear lamina in coordinating different signaling pathways during spermatogenesis.

Since the discovery that some human diseases are caused by mutations in A-type lamins, much effort has been devoted to deciphering the disease mechanism by focusing on the study of A-type lamins. Mice deleted of the gene encoding for A-type lamins die a few weeks after birth (Sullivan et al., 1999), whereas deleting either one or both genes encoding for lamin-B1 or lamin-B2 results in perinatal lethality (Coffinier et al., 2010; Kim et al., 2011; Vergnes et al., 2004). These observations have led to the prevailing but unproven belief that A- and B-type lamins have very distinct functions. Our findings demonstrate that A and B types have shared functions in the CySC lineage. This shared function could explain why the loss of B-type lamin results in strong defects in cyst and follicle cells that only express lamin-B. Therefore, our findings emphasize the need to consider the shared functions of A- and B-type lamins when interpreting the developmental or disease phenotypes caused by mutations in a specific lamin.

As an important node that connects chromatin in the nucleus to cytoskeleton in the cytoplasm, lamins may have a general role in integrating signaling pathways with gene regulation and cell morphogenesis in somatic cells that form the niche for stem or progenitor cells. Considering the broad roles of signaling pathways, the function of lamins in cell signaling that we report here could help to explain the diverse roles assigned to these nuclear proteins by previous in vitro studies (Dechat et al., 2008). We suggest that the function of lamins in a given cell type could differ depending on the signaling pathways used by the cell. Therefore, to reach a comprehensive understand of lamins, it is important to use tissue-specific deletion strategies. The effort to understand the role of lamins in different stem cell niches should also contribute toward the study of the disease mechanism of nuclear lamina-based tissue degeneration.

EXPERIMENTAL PROCEDURES

All mutant and transgenic *Drosophila* strains used in this study are described in detail in Supplemental Experimental Procedures. Different strains were raised at low density on standard cornmeal/molasses/agar fly food at 25°C unless stated otherwise. To rescue LAM depletion, we created UAS-*dERK*-NLS transgenic fly strains.

FLP/FRT-mediated mitotic recombination (Xu and Rubin, 1993) was used to generate *Lam* null mutant germline clones using the Lam^{D395} allele. To analyze the effect of LAM deletion on cyst cell morphology, we used the FRT "flip out" method (Pignoni and Zipursky, 1997). Testes containing LAM-depleted cyst

and (F) are nonspecific antibody labels. The hubs, GSCs, and CySCs are outlined with yellow, red, or blue dotted lines, respectively. Scale bars represent 10 μm.

(G and H) Electron micrographs of cyst cells revealing that LAM depletion did not affect the assembly of nucleopores in these cells. Both the face (G′ and H′, arrows) and side (G″ and H″, arrowheads) views of nucleopores are shown. N, nucleus; C, cytoplasm. Scale bars represent 0.1 μm.

(I and J) Depletion of either NUP153 (I) or MTOR (J) in the CySC lineage resulted in the accumulation of DAPI-bright early germ cells (green dashed lines) at the expense of DAPI-dim differentiated germ cells (red dashed lines) in testes, similar to those seen in LAM depletion. Asterisks represent hubs. Scale bars represent 50 μm.

(K) Quantification of the percentages of L3 gonads with mislocalized CySCs as judged by the close proximity of the Zfh-1+ nuclei to the hub. The colored bars show the number of CySCs near each hub. The *tj*-driven *Nup153* (n = 32) or *Mtor* RNAi (n = 28) caused a significant increase of gonads with mislocalized CySCs compared to the control *tj*-driven LacZ RNAi (n = 25). ***p < 0.001, Wilcoxon two-sample test.

(L) Quantification of the number of GSCs associated with the hub. The *tj*-driven *Nup153* (n = 17) or *Mtor* RNAi (n = 21) caused a significant decrease in the number of GSCs associated with the hub compared to the control *tj*-driven LacZ RNAi (n = 13). ***p < 0.001, Student t tests. Error bars represent SD.

See also Figure S5.

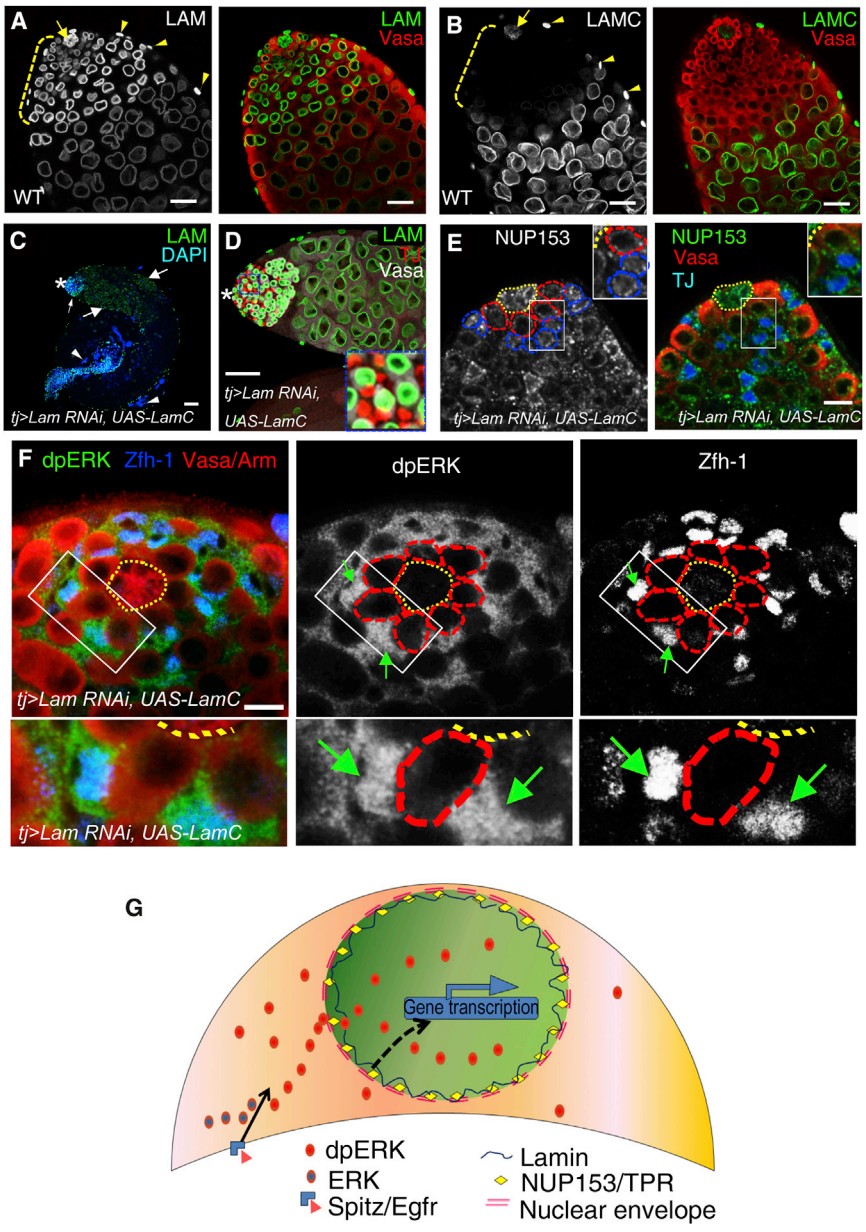

Figure 7. A Shared Function between A- and B-type Lamins in the CySC Lineage

(A and B) A strong and ubiquitous expression of LAM (A) in the wild-type adult testis compared to the undetectable LAMC in the CySCs, early cyst cells, and early germ cells (B) in the tip region (brackets). LAMC is expressed in the hub and basement muscle cells. Arrows represent hub cells. Arrowheads represent basement muscle cells. Scale bars represent 20 μm.

(C and D) Ectopic expression of *tj* > *LamC* cDNA in cyst cells resulted in strong (34 out of 56) or weak (22 out of 56) rescues of germ cell differentiation defects caused by the CySC-lineage-specific *Lam*-RNAi as judged by LAM (green) and DAPI (blue) staining. Small arrows, large arrows, and arrowheads in (C) indicate early germ cells, spermatocytes, and sperm bundles, respectively, in a strongly rescued testis. The tip region in (C) is enlarged in (D) with the inset showing cyst cells (red, lacking LAM) and germ cells (white, containing LAM). An example of a weak LAMC rescued testis and a control *tj*-driven *LacZ* cDNA expressed testis are shown in Figures S6E and S6F and Figures S6G and S6H, respectively. Asterisks represent hubs. Scale bar, 50 μm.

(E) Ectopic expression of *tj* > *LamC* cDNA in the CySC lineage resulted in a partial rescue of the NUP153 localization defect caused by *tj* > *Lam*-RNAi in a strongly rescued testis. An example of a testis with control *tj*-driven *LacZ* cDNA expression is shown in Figure S6I. The hubs, GSCs, and CySCs are outlined with yellow, red, or blue dotted lines, respectively. Scale bars represent 10 μm.

(F) The nuclear dpERK localization defect caused by the CySC lineage specific *Lam*-RNAi was partially rescued by the ectopic expression of *tj*-driven *LamC* cDNA in a strongly rescued L3 male gonad. Boxed areas in (F) are enlarged to the bottom. Green arrows point to CySCs with nuclear dpERK staining. Images of the control expression of *tj*-driven LacZ cDNA are shown in Figure S6J. Arm (red), Vasa (red), and Zfh-1 (blue) label the hub, the germline lineage, and the CySC lineage, respectively. The hub and GSCs are outlined with yellow and red dotted lines, respectively. Scale bar represents 10 μm.

(G) A model illustrating that LAM regulates the maintenance and differentiation of the GSC and transit-amplifying germ cells by controlling the localization of CySCs and cyst cell morphogenesis via the nucleoporin-dependent EGFR signaling.

See also Figure S6.

cells were dissected and processed for immunostaining 4–5 days after being heat shocked for 1 hr at 37°C at the 8-day pupal stage. GFP expression allowed the marking of the morphology of individual cyst cells. For more detailed procedures of clonal analyses and the RNAi-FRT flip out strategy used, please refer to Supplemental Experimental Procedures. All other techniques used, including immunofluorescence/electron microscopy, nuclear extract preparation, and immunoprecipitation, are also detailed in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes *Drosophila* strains, Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.05.003.

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